The Effects of HMBi on Nitrogen Fractions in Continuous Culture Fermenters

Thesis

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Abstract

2-hydroxy-4-(methylthio)-butanoic acid (HMB) has been used for years in the feed industry as a precursor for the limiting amino acid, methionine (Met). Because Met is one of the limiting amino acids in a dairy cow's diet, increasing the amount of Met absorbed from the small intestine should allow the other non-limiting amino acids (AA) to be converted to more milk protein and thereby decrease the elimination of ammonia-nitrogen (NH_3-N) in the waste. An isopropyl ester was attached to HMB (HMBi) to slow its degradation rate in the rumen to allow for more uptake by the rumen microorganisms. Some bacteria have the enzymes to add an amino group to HMB to make Met. We hypothesized that HMBi is converted to HMB more slowly, thus providing a more sustained source of Met to enhance microbial protein synthesis (MPS) and decrease NH_3-N excretion.

A continuous culture fermenter system was used in a 4 x 4 Latin square design using four fermenters and four periods. The fermenters were inoculated with rumen fluid from two Holstein dairy cows. The treatments were 1) control, 2) Met, 3) HMBi+Met, and 4) HMBi with the latter three all on the same molar basis The stable isotopes ^{13}C-HMBi, ^{13}C-Met, and ^{2}H-Met were infused to study bacterial metabolism.

After infusion of ammonium sulfate labeled with ^{15}N, four samples per period per fermenter were collected over 16 hr. I analyzed for N from bacteria, ammonia, and effluent and sent samples for ^{15}N analysis to determine efficiency of microbial N synthesis.

I found that HMBi tends to increase peptide N in the HMBi and HMBi+Met treatments and decreased NH_3-N for HMBi. In addition, molar percentage of propionate, isovalerate, and valerate were higher in the HMBi and HMBi+Met treatments. In contrast to our hypotheses, bacteria used preformed Met more efficiently and synthesized more Met when HMBi was fed,

diverting carbon away from volatile fatty acid (VFA) and adenosine triphosphate (ATP) synthesis.

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List of Abbreviations

AA	amino acids
BCVFA	branched chain volatile fatty acid
BUN	blood urea nitrogen
EMPS	efficiency of microbial protein synthesis
HMB	2-hydroxy-4-(methylthio)-butanoic acid
HMBi	isopropyl ester of 2-hydroxy-4-(methylthio)-butanoic acid
MCP	microbial crude protein
MPS	microbial protein synthesis
Met	methionine
N	nitrogen
NH3	ammonia
NH3-N	ammonia-nitrogen
RDP	rumen degraded protein
RUP	rumen undegraded protein
TCA	trichloroacetic acid
VFA	volatile fatty acid

Introduction

Bacteria use Met for incorporation into protein as well as a source of N and carbon (Brosnan et al., 2007). Met is an important molecule for prokaryotic and eukaryotic growth. Rumen bacteria are stimulated by peptides and AA, and because Met has functions beyond other AA as sources of N, carbon, and protein structure, it may positively affect the microbial growth of the rumen bacteria due to an increase in microbial protein production (Atasoglu et al., 1999).

The rumen environment is protected from low ammonia (NH_3) concentrations due to the recycling of blood urea nitrogen (BUN) which enables rumen bacteria to produce all the necessary AA for milk production (Firkins et al., 2007). The rumen bacteria are able to synthesize Met and incorporate it into bacterial protein (Or-Rashid et al., 2001). However, the supply may be insufficient because milk production from high producing dairy cows has been shown to be limited by Met and lysine (Rulquin et al., 2006). Supplementation of Met to dairy cows could stimulate growth of rumen bacteria and therefore the postruminal supply of Met to dairy cows. In cows Met supplementation has increased milk protein yield (Rulquin and Delaby, 1997).

This cycling of BUN is also indicative of NH_3-N precursors that may become more limiting than NH_3-N in dairy cows fed high amounts of rumen-degraded starch. Carbohydrate supply can influence the amount of NH_3-N that is converted into microbial protein. Also, there is evidence for an optimum range of rumen degraded starch above and below that which can have detrimental effects on total MPS as well as efficiency of microbial protein synthesis (EMPS) (Firkins et al., 2007).

When rumen bacteria degrade Met for carbon and N, the amount that escapes the rumen may not be adequate for milk production. Thus, Met analogs have been produced to decrease

degradation, thereby increasing the supply of Met to the cow as well as increasing the length of time the Met is supplied to the rumen bacteria. HMB and its isopropyl ester, HMBi, are Met analogs that have been designed for this purpose. HMBi has been shown to increase milk, milk fat, and protein yields and true protein percentage in cows that are fed a diet deficient in Met (Rulquin et al., 2006). HMB did not increase milk production or components in that study. In continuous cultures, HMB decreased the percentage of bacterial N originating from NH_3-N by about 13%, indicating that HMB is not simply a Met precursor (Noftsger et al., 2003). HMBi may be degraded more slowly by the rumen bacteria thereby providing a more steady supply of HMB to the bacteria and positively affect growth.

Protein requirements in dairy cows have been studied consistently as the understanding of MPS, rumen degradability, and nutrient requirements has improved. Microbial crude protein (MCP) is a major source of protein for milk production because it flows out of the rumen and into the abomasum. The AA profile of milk is very similar to the AA profile of rumen bacteria. The EMPS can have a major effect of milk production due to the effect on MCP yield. It is difficult to determine the actual flows of N fractions to the duodenum and omasum because these N fractions originate from rumen degradable protein (RDP) and N recycling.

Optimizing microbial growth through the supplementation of AA could result from a decrease in energy use from breaking down carbon sources and synthesizing the appropriate amino acids. Ives et al. (2002) report that the rumen bacterial AA pool size remained similar in cattle fed different diets. However, Bach et al. (2004) reported that the AA made from carbon skeletons varied by the diet fed. Some AA are more likely to be metabolized, but others are more likely to be incorporated into microbial protein by the rumen bacteria (Atasoglu et al., 2004).

Atasoglu et al. (2004) reported that supplying all the AA to rumen bacteria increased the bacterial protein synthesis. Synthesis could be measured by incorporation of N into microbial cells. The rumen bacteria that were supplemented with Met grew similarly to those with NH_3 only (Kajikawa et al., 2002). The study also showed that by removing certain amino acids - leucine, tryptophan, tyrosine, glutamate, Met, phenylalanine, and valine – from the supplement significantly decreased the growth stimulation.

Volatile fatty acid (VFA) synthesis in rumen bacteria is related to AA synthesis. After dietary carbohydrates enter glycolysis, pyruvate can be metabolized to VFA for ATP synthesis or pyruvate carbon can be converted to cellular compounds, including AA (which requires ATP). In addition,dietary AA can be degraded for VFA synthesis. Valine can be deaminated to isobutyrate, leucine to isovalerate, and isoleucine to 2-methylbutyrate. These VFA are growth factors for cellulolytic bacteria to use for long chain fatty acid synthesis (Van Soest, 1994). Moreover, phenylalanine is an important growth factor for some cellulolytic and other bacteria (Atasoglu et al., 2004).

Continuous culture fermenters allow for the substrate supply and the passage rate to be separated. Studies have shown that EMPS can be increased through increasing the passage rate. According to Firkins et al. (2007), the increase in efficiency is due to an increase in gene expression in the bacteria populations that are highly competitive for the substrate available or that have another strategy to out-compete other bacteria.

A dual-flow continuous culture system allows metabolites to escape at a constant rate so pool size is not disturbed. The samples of the bacterial populations from the continuous culture system is prone to less sampling error due to the large size of a rumen compared to the sample size and because we can measure flow directly rather than relying on indirect marker approaches.

In addition, the samples would be homogenous due to constant and equal agitation by the paddles. The continuous culture will allow isotopically labeled isotopes to be used in small amounts. If large amounts were used, it could possibly elicit an animal response that would not be linked to bacterial changes and would be cost-prohibitive.

Milk protein production increases as a result of postruminal infusion of Met (Pisulewski et al., 1996). This Met supplementation indicates that by increasing Met flow out of the rumen or by increasing total MCP flow to the rumen, production may be able to be increased. The Met requirement for high producing dairy cows in peak, early, and mid-lactation was determined when fed diets that were formed to meet or slightly exceed nutrient requirements. Duodenal infusion of 16g/day of Met during early lactation linearly increased (P=0.05) 3.5% fat-corrected milk, milk fat, and milk true protein. The increase in milk true protein percentage had a quadratic effect (P=0.04) during mid-lactation. In addition, there was a linear increase (P=0.04) in milk production due to Met supplementation. During peak lactation, infusion of 16g/day increased milk true protein production linearly (P=0.03). However, there was no quadratic effect, indicating that the Met requirement was not met for peak lactation. These results indicate that Met can be limiting in early, mid, and peak lactation for cows that are fed diets formulated to meet nutrient requirements. In addition, it shows that Met supplementation may increase milk protein production.

HMB is a Met hydroxyl analog that is fed to cows. Noftsger et al. (2003) showed that HMB in continuous culture fermenters tended to decrease the proportion of microbial nitrogen originating from NH_3-N by about 13%. The amount of nitrogen in MCP from NH_3 should not decrease if HMB was simply a Met precursor. Therefore, HMB probably has an effect of MPS from preformed AA other than just Met. HMBi might be degraded more slowly by the rumen

bacteria. Thus, it could provide a more steady supply of Met to the bacterial populations and positively affect growth. Through blood and milk true protein analyses, studies showed that HMBi is 50% undegraded in the rumen (Noftsger et al., 2005). The undegraded part is absorbed through the rumen wall where the isopropyl group is removed and HMB goes into the blood stream. The other 50% is broken down into HMB and isopropanol. This is supported by the HMBi and HMB recoveries through the omasum of 2.3% and 5.3% respectively (Noftsger et al., 2005). These results, however, do not support other studies that show large amounts of HMB passing from the rumen in the liquid to a postruminal supply of Met to the cow.

The effects of HMB and HMBi were compared by Rulquin et al. (2006) when fed to lactating dairy cows. By supplementing HMBi to a diet deficient in Met, milk protein yield and true protein percentage increased. HMB did not show an effect on milk production or milk components. St-Pierre and Sylvester (2005) showed that HMBi increased milk fat, milk, and milk protein yields when fed to cows in early lactation that are deficient in Met, but not in lysine. HMB, again, did not increase in milk production or milk components. Cows that were fed an HMBi supplemented diet of 17.5 g/day had a decrease in amount of urinary N excretion, meaning more N was recovered in the milk and therefore, gross N efficiency was increased by HMBi. Noftsger et al. (2005) compared the production effects of 0.10% HMB, 0.13% HMBi, and 0.88% $_{DL}$-Met. In the study, HMBi caused a higher protein production than Met, and HMBi and Met increased the milk protein percentage. The rumen digestibility of organic matter was increased in all the supplemented diets.

Materials & Methods

Collection of rumen fluid occurred on the morning of the first day. The rumen contents were taken from two different cows for a total collection of about eight liters, and filtered through four sheets of cheesecloth twice. The rumen fluid was then separated into the four different fermenter systems. During the experiment, the fermenters were fed and treated in increments of eight hours. I helped monitor the filtrate and overflow outflow rates, pH, temperature, and agitation. The filtrate outflow rate was kept at 12.5% per hour, and the overflow outflow rate was maintained at 5.5% per hour. We will be able to figure out the percent of degradation by a calculation of the rate of degradation per hour over the rate of degradation per hour plus the outflow rate:

$$\frac{k_{deg}}{k_{deg} + k_{out}} = \% \text{ degradation}$$

The goal of the experiment was to keep the pH within 6.0 and 7.0. If it ever fell below a pH of 6.0 for any length of time, the period was ended prematurely and restarted. The temperature was kept at a constant 39 degrees C, and the agitation was at 160 rpm.

The experimental design that was used was a 4x4 Latin square, and there were four different fermenters used with four different treatments. One treatment was a control while the other treatments were Met only, HMBi & Met, and HMBi only. The labeled HMBi and Met were only dosed six times during each period. There was a seven-day adaptation period to allow the bacteria to adapt to the fermenters and treatments. The labeled HMBi and methionine that were dosed allowed us to track what the bacteria utilized. When the dosing occurred, the regular treatments were still used, but they were reduced to account for the additional doses that were administered at the same time.

Labels were used in our experiment such as ^{13}C (heavy carbon), ^{2}H (heavy hydrogen, or deuterium), and ^{15}N (heavy nitrogen). The ^{13}C is a heavy carbon – increased by one mass unit due to an extra neutron. The most common form of carbon is the elemental carbon ^{12}C which contains an equal number of protons and neutrons. ^{2}H is heavy hydrogen, meaning that instead of having one proton and zero neutrons (which is how H is most commonly found); it contains one proton and one neutron, thus increasing the H by one mass unit. The final label that was used in the experiment was that of ^{15}N. The same concept applies for the isotopes of C and H. The ^{15}N has an extra neutron, thus making it heavier than the most common form ^{14}N.

To evaluate kinetics of degradation versus the uptake of HMBi into microbial cells, we used HMBi in which all of the HMB carbons were labeled with ^{13}C (*HMBi- ^{13}C). Met was labeled at the first carbon with ^{13}C (*Met- ^{13}C) or all three of the H's on the methyl were labeled with ^{2}H (*Met- ^{2}H). For each period, the HMBi fermenter was dosed at 25% *HMBi- ^{13}C and 75% HMBi, the HMBi and Met fermenter was dosed at 25% *HMBi- ^{13}C, 25% HMBi, 30% *Met- ^{13}C, and 20% Met, the Met fermenter was dosed at 30% *Met- ^{2}H, 30% *Met- ^{13}C, and 40% Met, and the control fermenter was just addition of distilled water. Treatments and doses were given based on a total equimolar basis and were always given right after feeding. By keeping it consistent within each period as well as among periods, the error should be reduced.

The Met and HMBi that were used for the dosing in each period were labeled. Upon doing the analysis of the data, the heavy carbon skeletons allow us to more easily analyze the metabolites – what the HMBi and Met are broken down into, and where they are incorporated. A metabolite of methionine is known to be the primary methyl group donor in metabolic reactions of bacteria, which is why we dosed a labeled Met that contains heavy hydrogen. We

can see which products are methylated by the metabolite by observing the heavy hydrogens on the methyl group that is donated.

The background samples for ^{15}N were taken on day four before the ^{15}N was introduced to the fermenters. ^{15}N was incorporated into all of the microbially synthesized amino acids. We collected four ^{15}N samples over a timeframe of 16 hr. The ^{15}N was used in order to make it easier to analyze how the bacteria utilize the N from NH_3 vs. AA. If bacteria take up preformed AA, the ^{15}N enrichment should decrease. Different components of nitrogen fractions include analyzing: peptide N, NH_3-N, bacterial N, as well as the total N (from effluent). TCA-soluble N was used to determine the peptide N by subtracting the ammonia from the value found for the TCA. TCA precipitates large peptides of proteins. The RDP and RUP will both be analyzed in our nitrogen fractions as well. In addition, ^{15}N allows us to measure the amount of MPS.

Table 1. Ingredients of Diet	
Ingredient	Base Diet
	% DM of Diet
Alfalfa pellets	50
Pelleted concentrate	50
Corn grain, ground	31
Dry distillers grains	6.5
Blood meal, ring-dried	0.5
Soybean hulls	10.8
Dicalcium phosphate	0.33
Limestone	0.065
MgO	0.13
Trace mineralized salt[1]	0.5
Vitamin A	0.012
Vitamin D	0.033
Vitamin E	0.059

[1]Contained 0.10% Mg, 38.08% Na, 58.0% Cl, 0.04% S, 5,000 mg/kg of Fe, 7,500 mg/kg of Zn, 2,500 mg/ kg of Cu, 6,000 mg/kg of Mn, 100 mg/kg of I, 60 mg/kg of Se, and 50 mg/kg of Co.

Table 2. Nutrient Composition of Diet	
Nutrient	% DM
NDF	34.9
SDF	23.6
CP[1]	14.2
RDP[2]	7.8
RUP[2]	6.4

[1] Found experimentally (Kjeldahl method)
[2] Calculated from book values NRC, 2001

Table 3. Nutrient digestibilities, nitrogen fluxes, and nitrogen partitioning for rumen bacteria in continuous cultures supplemented with HMBi[1], DL-methionine or both

	Treatment					Contrasts[2]		
	Con	Met	HMBi+Met	HMBi	SEM	CON vs. all	Linear	Quadratic
Digestibility (%)								
NDF	42.0	46.3	46.0	38.8	2.6	NS[3]	0.04	NS
ADF	48.4	46.6	51.8	47.3	3.6	NS	NS	NS
Hemicellulose	28.9	44.4	33.8	20.7	5.0	NS	0.01	NS
True OM	50.4	51.3	50.6	49.2	2.0	NS	NS	NS
Nitrogen flows (g/day)								
Ammonia N	0.36	0.39	0.39	0.32	0.03	NS	0.08	NS
NAN[4]	2.50	2.38	2.45	2.38	0.15	NS	NS	NS
Bacterial N	1.57	1.62	1.57	1.50	0.10	NS	NS	NS
Total N	3.15	3.10	3.18	2.97	0.15	NS	NS	NS
NANBN[5]	0.98	0.82	0.92	0.94	0.11	NS	NS	NS
Nitrogen Partitioning								
Ammonia N (mg/dl)	6.22	6.80	7.20	5.65	0.37	NS	0.07	0.08
TCA-soluble N (mg/dl)	11.4	12.1	12.3	11.8	0.37	0.06	NS	NS
Peptide N[6] (mg/dl)	5.17	5.28	5.14	6.13	0.27	NS	0.04	0.09
Bacterial N derived from NH_3-N, %	86.2	81.8	88.5	89.1	1.80	NS	0.02	NS
Bacterial N efficiency[7]	28.9	32.0	31.7	30.9	1.95	NS	NS	NS

[1]HMBi = 2-hydroxy-4-(methylthio) butanoic acid.
[2]All supplementation (HMBi, HMBi+MET, MET) vs. control, and linear and quadratic responses to the three supplements.
[3]NS= Not significant; *P*>0.10.
[4]NAN= Non-ammonia nitrogen.
[5]NANBN= Non-ammonia non-bacterial nitrogen = NAN – bacterial N
[6]Nitrogen soluble in 10% (vol/vol) trichloroacetic acid.
[7]Grams microbial N produced/kilogram OM truly digested.

Table 4. Volatile fatty acid production per day for rumen bacteria in continuous culture supplemented with HMBi[1], $_{DL}$-methionine or both

	Treatment					Contrasts[2]		
	Con	Met	HMBi+Met	HMBi	SEM	Con vs. all	Linear	Quadratic
Production (mmol/day)								
Acetate	119	118	116	111	5.11	NS[3]	0.06	NS
Propionate	35.7	36.1	32.4	31.9	1.24	0.01	<0.01	0.05
Isobutyrate	0.56	0.66	0.66	0.52	0.05	NS	0.05	NS
Butyrate	19.9	20.6	18.4	18.5	0.84	NS	NS	NS
Isovalerate	3.33	3.37	4.90	4.80	0.57	NS	0.08	NS
Valerate	4.06	3.97	4.81	4.90	0.25	0.01	<0.01	0.05
BCVFA[4]	3.88	4.02	5.56	5.32	0.6	NS	NS	NS
Total VFA	183	183	177	172	7	NS	0.02	NS
Acetate: Propionate	3.33	3.28	3.58	3.48	0.09	NS	0.08	0.06

[1]HMBi = 2-hydroxy-4-(methylthio) butanoic acid.
[2]All supplementation (HMBi, HMBi+MET, MET) vs. control.
[3]NS= Not significant; $P>0.10$.
[4]BCVFA= Branched chain VFA, which includes isobutyrate and isovalerate.

Results & Discussion

The diet fed during this experiment was formulated in order that it be limiting in RDP so microbial responses to Met could occur in cows that were fed diets formulated to decrease N excretion. The flows of effluent for the differing N partitions were not different by treatment as seen in Table 3. The concentration of NH_3-N tended to be linearly (P=0.07) and quadratically (P=0.08) affected. This is due to HMBi decreasing NH_3-N concentration compared to that of Met or HMBi+Met. NH_3-N might be increased by the supplementation of Met due to an increase in deamination. A decrease in NH_3-N due to HMBi supplementation might be caused by an increase in ammonia utilization for amination of HMBi to Met. An increase in amination of HMBi could additionally explain the increase in bacterial N as being derived from the HMBi+Met and HMBi treatments. Noftsger et al. (2003) showed a decrease in bacterial N from ammonia due to HMB in continuous culture. This could be due to increasing proteolysis if HMB was used as a carbon source or that it stimulates the uptake of preformed amino acids.

The concentration of TCA-soluble N tended (P=0.06) to be less than the average of the Met treatments (Table 3). The TCA-soluble N includes ammonia and peptides. However, the peptide N was affected linearly (P=0.04) and tended to be affected quadratically (P=0.09). This difference could be due to peptide concentration being greatest in the HMBi culture. The percentage of bacterial N that was derived from NH_3-N was increased (P=0.02) as HMBi replaced Met. Contrary to our original hypothesis, the increasing peptide N and decreasing NH_3-N concentrations with increasing HMBi supplementation appears to support a decrease in preformed amino N. The increase in percentage of bacterial N from NH_3-N in the HMBi treatment in relation to the control could indicate that HMBi was aminated to Met and the Met

was then incorporated into bacterial protein. However, since Met only makes up a small part of the protein in bacteria, our data indicate that other amino acids must have been synthesized de novo as well. The percentage of bacterial N from NH_3-N in the Met treatment was decreased in relation to the control indicating that the bacteria used the available preformed amino acids.

The effect of HMB and Met on VFA production is shown in Table 4. Butyrate and branched chain volatile fatty acids (BCVFA) were not affected by treatment; however, acetate tended ($P=0.06$) to decrease as HMBi replaced Met. Propionate was affected linearly ($P<0.01$) and quadratically ($P=0.05$) while averaging less ($P=0.01$) for the three treatments compared to the control showing that both amounts of HMBi supplementation decreased propionate production. Isobutyrate decreased linearly ($P=0.05$) with increasing HMBi, even though isobutyrate was higher for Met and HMBi+Met in relation to the control. Isovalerate tended to increase linearly ($P=0.08$) with increasing HMBi. Valerate was linearly ($P<0.01$) and quadratically ($P=0.05$) affected by treatments and all the treatments were greater ($P=0.01$) than control. This indicates that valerate was increased for both HMBi+Met and HMBi. The total VFA production was decreased ($P=0.02$) as increasing HMBi replaced Met. The acetate to propionate ratio tended to be affected linearly ($P=0.08$) and quadratically ($P=0.06$), thus indicating increasing ratios for HMBi+Met and HMBi rather than for Met. These changes in VFA production due to supplementation of Met as HMBi or Met itself could indicate a change in activity of the present bacteria populations. This could be achieved by some populations incorporating preformed AA into bacterial protein, and others by using AA as N and carbon sources. HMBi appeared to change metabolism or benefit the bacteria populations that aminate carbon skeletons readily by utilizing the resulting Met.

Conclusions & Further Work

The activity of the bacteria populations might be increased due to Met supplementation. HMBi appeared to be aminated to Met by rumen bacteria, but then incorporated into the bacterial protein at a very slow rate (Fowler, C.M.S. Thesis). The supplementation of HMBi increased the flow of peptides but decreased the net production of total VFA. This indicates that carbon was used for the synthesis of AA instead of using preformed AA. Due to the changes in VFA production, the supplementation of Met as HMBi or Met itself might change the activity of the rumen bacteria populations or their activity.

In order to further explore the effect of HMBi, the rate of incorporation of Met from Met and HMBi into bacterial protein, the rate of production of Met from HMBi should be measured. More extensive analyses of microbial populations would verify that the supplementation resulted in a change in metabolism rather than a change in bacteria populations.

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